JC16 Rec'd PCT/PTO SEP 10 2001

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 09/936543	INTERNATIONAL APPLICATION NO. PCT/EP00/01797	ATTORNEY'S DOCKET NUMBER 112843-031

21. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

	CALCULATIONS	PTO USE ONLY
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1,000.00**		
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but Internation Search Report prepared by the EPO or JPO **$860.00**		
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☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**		
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$860.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	23 - 20 =	3	x $18.00	**$54.00**	
Independent claims	6 - 3 =	3	x $78.00	**$234.00**	
Multiple Dependent Claims **(check if applicable).**			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$1,148.00**	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) **(check if applicable).** ☐				**$0.00**	
SUBTOTAL =				**$1,148.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$1,148.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐				**$0.00**	
TOTAL FEES ENCLOSED =				**$1,148.00**	
				Amount to be: refunded	$
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☒ A check in the amount of **$1,148.00** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. in the amount of to cover the above fees. A duplicate copy of this sheet is enclosed.

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NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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REGISTRATION NUMBER

September 10, 2001
DATE

09/936543

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Reniero et al.
Appl. No.:	PCT/EP00/01797
Filed:	March 2, 2000
Title:	LACTIC ACID BACTERIA STRAINS CAPABLE OF PREVENTING DIARRHOEA
Art Unit:	Unknown
Examiner:	Unknown
Docket No.:	112843-031

Assistant Commissioner for Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

Sir:

Please amend the above-identified patent application as follows:

In the Claims:

Please amend Claims 3 and 6-11 as follows:

3. (Once Amended) The lactobacillus according to claim 1 which is selected from the group consisting of Lactobacillus rhamnosus and Lactobacillus paracasei.

6. (Once Amended) A method for preparing an ingestable support material comprising the step of using lactic acid bacterium belonging to the genus Lactobacillus capable of adhering to and essentially colonizing the intestinal mucosa and capable of preventing infection of intestinal epithelial cells by rotaviruses.

7. (Once Amended) The method according to claim 6, wherein the Lactobacillus strain is contained in the ingestable support material in an amount from about 10^5 cfu / g to about 10^{12} cfu / g support material.

8. (Once Amended) The method according to claim 6 wherein the ingestable support material is a food composition selected from the group consisting of milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, and infant formulae.

9. (Once Amended) A method for the treatment of a disorder associated with diarrhoea comprising the step of administering to a patient suffering a disorder associated with diarrhoea lactic acid bacterium belonging to the genus Lactobacillus capable of adhering to and essentially colonizing the intestinal mucosa and capable of preventing infection of intestinal epithelial cells by rotaviruses.

10. (Once Amended) A pharmaceutical composition comprising a lactic acid bacterium belonging to the genus Lactobacillus capable of adhering to and essentially colonizing the intestinal mucosa and capable of preventing infection of intestinal epithelial cells by rotaviruses.

11. (Once Amended) The composition according to claim 10, which is selected from the group consisting of milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formulae, tablets, liquid bacterial suspensions, dried oral supplement, liquid oral supplement, dry tube feeding and liquid tubefeeding.

Please add newly submitted claims 12-23 as follows:

12. The pharmaceutical composition according to claim 10 wherein the Lactobacillus is capable to grow in the presence of up to 0.4% bile salts.

13. The pharmaceutical composition according to claim 10 wherein the Lactobacillus is selected from the group consisting of Lactobacillus rhamnosus and Lactobacillus paracasei.

14. The pharmaceutical composition according to claim 13 wherein the Lactobacillus is Lactobacillus paracasei.

15. The pharmaceutical composition according to claim 13 wherein the Lactobacillus paracasei strain is Lactobacillus paracasei CNCM I-2116.

16. The method of claim 9 wherein the lactic acid bacterium comprises an ingestable support material.

17. The method according to claim 9 wherein the Lactobacillus strain is contained in the support material in an amount from about 10^5 cfu / g to about 10^{12} cfu / g support material.

18. A method for preventing a disorder associated with diarrhea comprising the steps of administering a lactic acid bacterium belonging to the genus Lactobacillus capable of adhering to and essentially colonizing the intestinal mucosa and capable of preventing infection of intestinal epithelial cells by rotaviruses to a patient at risk of diarrhea.

19. The method according to claim 18 wherein the lactic acid bacterium is part of a composition selected from the group consisting of milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, and infant formula.

20. A food comprising a lactic acid bacterium belonging to the genus Lactobacillus capable of adhering to and essentially colonizing the intestinal mucosa and capable of preventing infection of intestinal epithelial cells by rotaviruses

21. The food of claim 20 which is selected from the group consisting of milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formulae, tablets, liquid bacteria suspensions, dried oral supplement, liquid oral supplement, dry tube feeding and liquid tubefeeding.

22. The food of claim 20 wherein the Lactobacillus is capable of growing in the presence of up to 0.4% bile salts.

23. The food of claim 20 wherein the Lactobacillus is selected from the group consisting of Lactobacillus rhamnosus and Lactobacillus paracasei.

REMARKS

This Preliminary Amendment is submitted in the above-identified patent application. Pursuant to the Preliminary Amendment Claims 3 and 6-11 have been amended and newly submitted Claims 12-23 have been added. This Preliminary Amendment does not add new matter. Further, Applicants note that the Preliminary Amendment is not being made for purposes of patentability but, merely to comport the claims to U.S. practice and/or to add additional claims. Applicants do not intend to disclaim any subject matter in view of the amendments.

Attached hereto is a marked-up version of the changes made to the claims by the current amendment. The attached page is captioned **"Versions with Markings to Show Changes Made."**

Respectfully submitted,

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BY

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Claims:

Claims 3 and 6-11 have been amended as follows:

3. (Once Amended) The lactobacillus according to claim 1 [or claim 2], which is selected from the group consisting of Lactobacillus rhamnosus [or] and Lactobacillus paracasei.

6. (Once Amended) A method for preparing [use of a lactic acid bacterium according to any of the preceding claims for the preparation of] an ingestable support material comprising the step of using lactic acid bacterium belonging to the genus Lactobacillus capable of adhering to and essentially colonizing the intestinal mucosa and capable of preventing infection of intestinal epithelial cells by rotaviruses.

7. (Once Amended) The method [use] according to claim 6, wherein the Lactobacillus strain is contained in the ingestable support material in an amount from about 10^5 cfu / g to about 10^{12} cfu / g support material.

8. (Once Amended) The method [use] according to [any of the claims] claim 6 [or 7] wherein the ingestable support material is a food composition selected from the group consisting of milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, and infant formulae.

9. (Once Amended) A method [The use according to any of the claims 5 to 7, wherein the support material used] for the treatment [and/or prophylaxis] of a disorder[s] associated with diarrhoea comprising the step of administering to a patient suffering a disorder associated with diarrhoea lactic acid bacterium belonging to the genus Lactobacillus capable of adhering to and essentially colonizing the intestinal mucosa and capable of preventing infection of intestinal epithelial cells by rotaviruses.

10. (Once Amended) A [Food or] pharmaceutical composition comprising a [containing] lactic acid bacterium belonging to the genus Lactobacillus capable of adhering to and essentially colonizing the intestinal mucosa and capable of preventing infection of intestinal epithelial cells by rotaviruses [at least once lactic acid bacterium according to any of the claims 1 to 4].

11. (Once Amended) The [composition] method according to claim [10,] 9 wherein the lactic acid bacteria is in a composition which is selected from the group consisting of [from] milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formulae, tablets, liquid bacterial suspensions, dried oral supplement, [wet] liquid oral supplement, dry tube feeding and [or] liquid [wet] tubefeeding.

Claims 12-23 were added.

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LACTIC ACID BACTERIA STRAINS CAPABLE OF PREVENTING DIARRHOEA

The present invention pertains to novel microorganisms of the family Lactobacillaceae, especially to microorganisms of the genus Lactobacillus, that are useful in preventing or treating diarrhoea. In particular, the present invention relates to the use of said microorganisms for the preparation of an ingestable support and to a composition containing the same.

Organisms that produce lactic acid as a major metabolic component have been known for a long time. These bacteria may be found in milk or in milk processing factories, respectively, living or decaying plants but also in the intestine of man and animals. These microorganisms, summarized under the term "lactic acid bacteria", represent a rather inhomogeneous group and comprise e.g. the genera Lactococcus, Lactobacillus, Streptococcus, Bifidobacterium, Pediococcus etc..

Lactic acid bacteria have been utilized as fermenting agents for the preservations of food taking benefit of a low pH and the action of fermentation products generated during the fermentative activity thereof to inhibit the growth of spoilage bacteria. To this end, lactic acid bacteria have been used for preparing a variety of different foodstuff such as cheese, yogurt and other fermented dairy products from milk.

Quite recently lactic acid bacteria have attracted a great deal of attention in that some strains have been found to exhibit valuable properties to man and animals upon ingestion. In particular, specific strains of Lactobacillus or Bifidobacterium have been found to be able to colonize the intestinal mucosa and to assist in the maintenance of the well-being of man and animal.

In this respect, EP 0 768 375 discloses specific strains of the genus Bifidobacterium, that are capable to become implanted in the intestinal flora and may adhere to intestinal cells. These Bifidobacteria are reported to assist in immunomodulation, being capable to competitively exclude adhesion of pathogenic bacteria to intestinal cells, thus assisting in the maintenance of the individual's health.

During the last few years research has also focused on the potential use of lactic acid bacteria as probiotic agents. Probiotics are considered to be viable microbial preparations which promote the individual's health by preserving the natural microflora in the intestine. A microbial preparation may be commonly accepted as a probiotic in case the effectual microbes thereof and their mode of action are known. Probiotics are deemed to attach to the intestine's mucosa, colonize the intestinal tract and likewise prevent attachment of harmful microorganisms thereon. A crucial prerequisite for their action resides in that they have to reach the gut's mucosa in a proper and viable form and do not get destroyed in the upper part of the gastrointestinal tract, especially by the influence of the low pH prevailing in the stomach.

In this respect, WO 97/00078 discloses a specific strain, termed Lactobacillus GG (ATCC 53103), as such a probiotic. The microorganism is particularly employed in a method of preventing or treating food induced hypersensitivity reactions in that it is administered to a recipient together with a food material that has been subjected to a hydrolysis treatment with pepsin and/or trypsin. The Lactobacillus strain selected is described as exhibiting adhesive and colonizing properties and showing a protease enzyme system, so that the protein material contained in the foodstuff to be administered is further hydrolysed by means of proteases secreted by the specific Lactobacillus strain. The method discussed in this document shall eventually result in the uptake of protein material by the gut that does not show a substantial amount of allergenic material anymore.

Further, in EP 0 577 903 reference is made to the use of such lactic acid bacteria having the ability of replacing Heliobacter pylori, the acknowledged cause for the development of ulcer, in the preparation of a support intended for the therapeutic or prophylactic treatment of an ulcer associated with the action of Heliobacter pylori.

In knowledge of the valuable properties particular strains of lactic acid bacteria may provide there is a desire in the art for additional lactic acid bacterial strains that are beneficial to the well being of man and/or animal.

Consequently, a problem of the present invention is to provide additional bacterial strains that exhibit new properties beneficial for man and/or animals.

The above problem has been solved by providing novel microorganisms, namely lactic acid bacteria, belonging to the genus Lactobacillus having the traits of being capable to adhere to and essentially colonize the intestinal mucosa and to prevent infection of intestinal epithelial cells by rotaviruses.

According to a preferred embodiment the Lactobacillus strains are capable to grow in the presence of up to 0.4 % bile salts, so that they may easily pass the gastrointestinal tract and stay essentially active.

According to another preferred embodiment the lactic acid bacterium is selected from the group consisting of Lactobacillus rhamnosus or Lactobacillus paracasei, preferably Lactobacillus paracasei, and is more preferably Lactobacillus paracasei CNCM I-2116.

The microorganisms of the present invention have been shown to exhibit the following properties, they are gram positive, catalase negative, NH_3 form arginine negative and CO_2 production negative. They produce L(+) lactic acid, are capable to

grow in the presence of bile salts in a concentration of up to about 0.4 % and may essentially prevent infection of epithelial cells by rotaviruses.

The novel microorganisms may be used for the preparation of a variety of ingestable support materials, such as e.g. milk, yogurt, curd, fermented milks, milk based fermented products, fermented cereal based products, milk based powders, infant formulae and may be included in the support in an amount of from about 10^5 cfu / g to about 10^{11} cfu / g. For the purpose of the present invention the abbreviation cfu shall designate a "colony forming unit" that is defined as number of bacterial cells as revealed by microbiological counts on agar plates.

The present invention also provides a food or a pharmaceutical composition containing at least one of the Lactobacillus strains having the above traits.

For preparing a food composition according to the present invention at least one of the Lactobacillus strains according to the present invention is incorporated in a suitable support, in an amount of from about 10^5 cfu / g to about 10^{11} cfu / g, preferably from about 10^6 cfu / g to about 10^{10} cfu / g, more preferably from about 10^7 cfu / g to about 10^9 cfu / g.

In case of a pharmaceutical preparation the product may be prepared in forms of tablets, liquid bacterial suspensions, dried oral supplements, wet oral supplements, dry tube feeding or a wet tube feeding etc., with the amount of Lactobacillus strains to be incorporated therein being in the range of up to 10^{12} cfu / g, preferably from about 10^7 cfu / g to about 10^{11} cfu / g, more preferably from about 10^7 cfu / g to about 10^{10} cfu / g .

The activity of the novel microorganisms in the individual's intestine is naturally dose dependent. That is, the more the novel microorganisms are incorporated by means of ingesting the above food material or the pharmaceutical composition the higher the

protective and/or curing activity of the microorganisms. Since the novel micro-organisms are not detrimental to mankind and animals and have eventually been isolated from baby feces a high amount thereof may be incorporated so that essentially a high proportion of the individual's intestine will be colonized by the novel microorganisms.

In the figures,

Fig. 1 shows a scheme illustrating the cell culture screening for assessing rotaviral protective properties of bacterial strains.

Fig. 2 shows the acidification of the L. casei strain CNCM I-2116 (termed ST11) in different growth media.

Fig. 3 shows the survival rate of the L. casei strain ST11 at 10 °C measured during 30 days.

Fig. 4 shows the mRNA pattern of IL-12 and IL-10 in mouse adherent cells derived from bone marrow after incubation of the cells with serial dilutions of ST11.

Fig. 5 shows the outcome of Th2 differentiation as resulting in decreased IL-4 production.

During the extensive studies leading to the present invention the inventors have investigated baby feces and isolated a variety of different bacterial strains therefrom. These strains were subsequently examined for their capability to prevent infection of epithelial cells with rotaviruses that are known to cause diarrhoea.

Several bacterial genera comprising Lactobacillus, Lactococcus, Streptococcus were screened for their rotavirus inhibitory properties. The tests for the inhibitory property

were essentially performed with three rotavirus serotypes representing the major etiological agents of human viral diarrhoea (serotypes G1, G3 and G4).

The various lactic acid bacteria were grown in a suitable medium, such as MRS, Hugo-Jago or M17 medium at temperatures of from about 30 to 40°C corresponding to their optimal growth temperature. After reaching stationary growth the bacteria were collected by centrifugation and resuspended in physiological NaCl solution. Between the different tests the bacterial cells were stored frozen (-20°C).

The various rotavirus stocks were prepared by infection of confluent cell monolayers. The rotaviruses were incubated before infection. The cells were infected with 20 tissue culture infectious doses.

For assessing anti-rotaviral properties two different protocols were applied. According to one protocol the various bacterial strains were examined for their direct interaction with the rotavirus while in the second protocol the bacteria were screened for those strains that interact with cellular rotavirus receptors.

The first protocol involved contacting the respective bacterial suspension each with a different rotavirus strain and incubating in suitable media. Subsequently, the virus-bacterium mixture was applied to a monolayer of cells of the human undifferentiated colon adenoma cells HT-29 and incubation was continued. Virus replication was then assayed.

The second protocol involved incubating the respective bacterial suspension first together with a monolayer of cells of the human undifferentiated colon adenoma cells HT-29 and adding the virus subsequently. After continued incubation virus replication was assayed.

Rotavirus replication may easily be assessed by histo-immunological staining of rotavirus proteins in infected cells.

A rotavirus inhibitory effect was attributed to a given bacterium when the number of infected cells was reduced by 90% in the cell culture inoculated with rotavirus plus the indicated bacteria in comparison with cells inoculated only with rotavirus.

Out of a total of 260 different bacterial strains primarily isolated merely 9 could be shown to essentially inhibit rotaviral replication. The different bacteria were ascertained to belong to the genus Lactobacillus subspecies rhamnosus or paracasei. One strain, termed Lactobacillus casei ST11, that has been deposited in accordance with the Budapest Treaty and has received the deposit numbers NCC 2461 (I-2116), has been shown to be extremely effective in preventing infection of human cells by rotavirus. Moreover, this particular strain shows excellent growing properties as may be shown by acidification in different media. The strain also shows good performance as regards the survival rate during storage at low temperatures of about 10 °C, which makes it an excellent candidate for being included in food stuff or pharmaceutical compositions to be stored at refrigerator conditions.

In addition to the above finding it could also be shown that the strains surprisingly also exhibit anti-allergenic properties in that said strains have an impact on the synthesis of different immunological mediators.

It is generally acknowledged that humoral immune responses and allergic reactions are mediated by $CD4^+$ T cells bearing the type 2 phenotype (Th2). Th2-cells are characterized by the production of high levels of interleukin 4 (IL-4), a cytokine required for the secretion of IgE, which is the major antibody class involved in allergic reactions.

The differentiation of Th2 cells is impaired by IFN-γ, a particular cytokine that arises from the mutually exclusive Th1 subset of $CD4^+$ T cells. Said Th1 cells are in turn strongly induced by interleukin 12 (IL-12). In contrast thereto IL-10, another cytokine, has been shown to have a strong suppressing impact on the proliferation of Th1 cells and is therefore deemed to play a role in immunosupressive mechanisms.

In summary, both IL-12 and IL-10 have strong modulatory effects on $CD4^+$ T cell development by influencing the development of the Th1 subset. IL-12 is a key regulatory cytokine for the induction of Th1 differentiation and thus inhibits the generation of Th2 responses. A major pathway for inhibition of Th2 cells is therefore seen in the stimulation of IL-12 synthesis by accessory cells.

It is well known that some components of gram negative bacteria, such as LPS, induce high levels of IL-12 in adherent cells, such as macrophages and dendritic cells. Consistently, it has been found that gram negative bacteria can strongly bias $CD4^+$ T cell differentiation towards the Th1 phenotype.

The microorganism ST11 as an example of the Lactobacillus strains of the present invention has been tested for a potential role in the induction of cytokines involved in the regulation of $CD4^+$ T cell differentiation. In particular, the effect of ST11 on the phenotype of $CD4^+$ T cells undergoing Th2 differentiation has been studied.

In this respect the capacity of ST11 to induce the synthesis of mRNA encoding these two regulatory cytokines in mouse adherent cells derived from bone marrow was compared with 4 other strains of Lactobacilli and with a control of gram negative bacteria (E.coli K12). The mRNA was measured by semi-quantitative RT-PCR after 6 hours of incubation of the cells with serial dilutions of bacteria ranging from 10^9 to 10^7 cfu/ml.

Although all strains of Lactobacillus could induce transcription of IL-12 mRNA to a certain degree, ST11 could be shown to be the strongest inducer, since as a strong PCR signal could be detected even at the lowest bacterial dose. In fact, the capacity of ST11 to induce IL-12 mRNA transcription was as strong as that of E. coli. Induction of IL-10 mRNA was in general weaker than for IL-12 mRNA, as only at higher bacterial doses a signal could be detected. Nevertheless, ST11 was the strongest inducer of IL-10 mRNA, as compared to the other lactobactilli and the E.coli control.

Thus, ST11 is deemed to be efficient in inducing immunoregulatory cytokines involved in $CD4^+$ T cell differentiation. Its strong capacity to induce IL-12 makes it a candidate to inhibit Th2 responses and its measurable IL-10 induction may prevent inflammatory responses.

In addition to the above finding it was also determined whether ST11 had an inhibitory effect on $CD4^+$ T cells undergoing Th2 differentiation and a positive effect on Th1 functions. A well established cell differentiation culture system was utilized, where precursor $CD4^+$ T cells were polyclonally activated and modulated to undergo either Th1 or Th2 differentiation, depending on the type of co-stimuli provided in the culture medium. Th1/Th2 differentiation was induced during a 7-days primary culture, after which the cells were then restimulated for 2 days in a secondary culture containing medium alone and acquisition of a specific phenotype (Th1 or Th2) was assessed by measuring the types of cytokines produced in the supernatant (IFN-γ vs. IL-4).

It is known that precursor $CD4^+$ T cells from mice of the BALB/c background preferentially differentiate to predominant Th2 phenotype (high IL-4, low IFN-γ in the 2ry culture supernatants) after activation under neutral conditions (medium alone in the 1ry culture). This phenotype could be completely reverted to a Th1 pattern

(high IFN-γ, low IL-4) upon addition of a blocking monoclonal antibody to IL-4 in the 1ry culture.

To investigate a potential role for ST11 on Th2 inhibition, purified precursor $CD4^+$ T cells from BALB/c mice were activated in the presence of bone marrow adherent cells as accessory cells during the 1ry culture. These cells were co-cultured either in medium alone, or in the presence of 1 mg/ml LPS, or 10^8 cfu/ml ST11, or 10^8 cfu/ml of another Lactobacillus. After this time, the cells were washed and $CD4^+$ T cells were purified once again and restimulated in the 2ry culture in medium alone. Cytokines produced by the differentiated $CD4^+$ T cells were measured after 2 days. As expected, cells differentiated in the presence of medium alone displayed a dominant Th2 phenotype. Addition of ST11 to the 1ry cultures strongly modulated the outcome of Th2 differentiation, as it resulted in an 8-fold decrease in IL-4 production. This inhibition was of similar magnitude as that observed in cultures derived from cells differentiated in the presence of LPS. In contrast, the other Lactobacillus strain had no measurable impact on IL-4 levels. Interestingly, IFN-γ levels were not increased upon addition of ST11 in the 1ry cultures.

In summary, ST11 specifically impaired IL-4 production by $CD4^+$ T cells undergoing Th2 differentiation, but did not significantly increase IFN-γ secretion. The fact that ST11 does not increase IFN-γ production may be due to its capacity to induce IL-10 with the consequence that it may keep a low inflammatory impact despite its anti-Th2 activity.

In consequence, it could be shown that ST11 is a Lactobacillus strain that has a good anti-Th2 profile which makes it an excellent candidate for its use as a bacterium with anti-allergic, probiotic activity.

The present invention will now be described by way of example.

Media and solutions:

MRS (Difco),

Hugo-Jago (Tryptone Difco 30 g/l, Yeast Extract Difco 10 g/l, Lactose Difco 5 g/l, KH_2PO_4 5 g/l, Beef Extract Difco 2 g/l, agar Difco 2 g/l)

M17 (Difco)

M199 (Seromed)

Ringer solution (Oxoid)

PBS (NaCl 8g/l, KCl 0.2 g/l, Na_2HPO_4 1.15 g/l, KH_2PO_4 0.2 g/l))

Tryptose phosphate broth (Flow)

Trypsin-EDTA solution (Seromed)

Human rotavirus Wa (G1 serotype) and simian rotavirus SA-11 (G3 serotype) were obtained from P.A. Offit, Children's Hospital of Philadelphia, U.S.A. The DS-1xRRV reassortant virus was obtained from A. Kapikian, NIH Bethesda, U.S.A. The serotype 4 human rotavirus Hochi was obtained from P. Bachmann, University of Munich, Germany.

Example 1

Isolation of lactic acid bacteria from baby feces

Fresh feces were harvested from diapers of 16 healthy babies 15 to 27 days old. 1 g of fresh feces was placed under anaerobic conditions for transportation to the laboratory and microbiological analyses were run within 2 hours from sampling by serial dilutions in Ringer solution and plating on selective media. MRS agar plus antibiotics (phosphomycine 80 µg/ml, sulfamethoxazole 93 µg/ml, trimethoprime 5µg/ml) incubated at 37°C for 48 hours was used to isolate lactic acid bacteria. Colonies were randomly picked up and purified. Physiological and genetic characterisation was performed on the isolates.

Example 2

Testing of strains in cell culture for anti-rotaviral activity

Several bacterial genera comprising Lactobacillus, Lactococcus, Streptococcus were selected and tested for members which showed anti-rotavirus activity in the cell culture inhibition test. The genus Lactococcus was represented by a single species (Lc. lactis) consisting of two subspecies (Lc. lactis supsp. lactis and cremoris). A total of 30 strains were tested. The Streptococcus genus was represented by a single species (S. thermophilus) with 45 strains. The Leuconostoc and Propionibacterium genus were only represented by a single species (6 strains), while the Enterococcus and Staphylococcus genus was represented by two species each and a total of 17 strains.

In total, 260 bacterial strains were tested for rotavirus inhibitory activity.

1st protocol:

30 µl of bacterial suspension (containing at average $3x10^6$ bacteria) were mixed with 70 µl M199 medium supplemented with 10% tryptose phosphate broth (Flow) and 5% trypsin-EDTA solution (Seromed) (1 : 4 diluted in the case of HT-29 cells) and 100 µl of virus in supplemented M199 medium. The virus-bacterium mixture was incubated for 1 hour at 4°C and for 1 hour at 37°C. Cells of the human undifferentiated colon adenoma cells HT-29 growing as a confluent monolayer in 96-well microtiter plates were washed three times with phosphate-buffered saline (PBS ; pH 7.2). The virus-bacterium mixture was applied to the cells and the microtiter plates were incubated for 18 h in a CO_2 incubator (Heraeus). Virus replication was assayed as described below.

2nd protocol:

30 µl of the bacterial suspension (supra) were mixed with 70 µl M199 medium supplemented with 10% tryptose phosphate broth (Flow) and 5% trypsin-EDTA

solution (Seromed) (1 : 4 diluted in the case of HT-29 cells) and applied directly on the cells in the microtiter plates. After one hour incubation at 37°C 100 μl of virus in supplemented M199 medium was added to the cells in the microtiter plates. The incubation was continued for 18 h in a CO_2 incubator (Heraeus). Virus replication was assayed as described below.

The rotavirus replication was assessed by histo-immunological staining of rotavirus proteins in infected cells as described hereafter.

One day after infection, the cell culture medium was discarded from the microtiter plates and the cells were fixed with absolute ethanol for 10 min. Ethanol was discarded, and the plates were washed three times in PBS buffer. Then 50 μl of an anti-rotavirus serum (mainly directed against VP6 protein), produced in rabbits (obtained from the ISREC University of Lausanne) and diluted 1 :2000 in PBS was added to each well and incubated for 1 h at 37°C with a cover slip to prevent desiccation of the wells. The antiserum was discarded afterwards and the plates were washed three times with PBS. Then 50 μl of anti-rabbit immunoglobulin G (IgG) antiserum produced in goats and coupled to peroxidase (GAR-IgG-PO; Nordic) were added at a dilution of 1 : 500 in PBS to each well and the plates were incubated for 1 hour at 37 °C. The serum was discarded and the plates were once again washed three times with PBS. Then 100 μl of the following substrate mixture was added to each well : 10 ml of 0.05 M Tris-hydrochloride (pH 7.8), 1 ml of H_2O_2 (30% suprapur, diluted 1 :600 in H_2O ; Merck) and 200 μl of 3-amino-9-ethylcarbazole (0.1 g/10 ml of ethanol stored in 200 μl aliquots at −80 °C ; A-5754 ; Sigma). The plates were incubated for at least 30 min at room temperature. The substrate was discarded and the wells were filled with 200 μl of H_2O to stop the reaction. Infected cell foci were counted with an inverted microscope (Diavert ; Leitz).

Only very few bacterial strains interacted with rotaviruses. Merely 9 out of the 260 bacterial cells primarily selected inhibited rotavirus replication in at least one

protocol. Lactobacillus paracasei NCC 2461 (ST11) showed an extremely high activity against Serotype 1 Rotavirus, Serotype 3 rotavirus SA-11 and Serotype 4 rotavirus Hochi.

Example 3

Properties of ST11

ST 11 has been subjected to incubation in simulated gastric juice. The simulated gastric juice was prepared by suspending pepsin (3 g/l) in sterile saline (0.5% w/v) and adjusting the pH to 2.0 and pH 3, respectively, with concentrated HCl. ST 11 has been grown in varying amounts in the above media and the resistance of the microorganisms has been determined.

The results are summarized in table I below .

Table I

pH	cfu/ml at T 0	cfu at T 1min	Cfu at T 15	cfu at T 30	cfu at T 60
2.0	2.0×10^9	1.8×10^9	1.2×10^9	3.7×10^8	7.0×10^3
3.0	2.0×10^9	1.9×10^9	1.7×10^9	1.7×10^9	8.4×10^8

ST11 has the following properties as defined according to methods disclosed in the genera of lactic acid bacteria, Ed. B.J.B. Wood and W.H. Holzapfel, Blackie A&P.

- gram positive,
- catalase negative,
- NH_3 form arginine negative
- CO_2 production negative
- production of L(+) lactic acid
- growth in the presence of bile salts in a concentration of up to about 0.4 %.

Example 4

Growth of ST 11 under different conditions

ST11 was incubated at 37 °C in tomato based medium (4% tomato powder rehydrated in distilled water) supplemented with sucrose (0, 0.5, 1 or 2%) or soya peptone (0.5%) or glucose (0.5%) for different periods of time. The results are shown in fig. 2.

ST11 was further added in an amount of 2.5 % to a medium composed of rice flour (3%), wheat flour (2%) and sucrose (3%) and incubated at 37°C until a pH of 4.4 was reached. After cooling the product was packed with or without addition of vit.C and stored at 10°C.

Example 5

Induction of IL-12 and IL10- mRNA synthesis in mouse adherent cells by ST11

Bone marrow cells were isolated from the femur and tibia of 8 week-old specific pathogen-free C57BL/6 mice and were incubated at a concentration of $2x10^6$ cells/ml in RPMI medium (Gibco) containing 10% fetal bovine serum, 1 mM L-Glutamine, 12 mM Hepes, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin (all reagents from Gibco) for 12 hours at 37°C in a 5% CO_2 atmosphere. Non-adherent cells were discarded by 3 consecutive washes with warm culture medium and the remaining adherent cells were harvested and incubated at a concentration of 10^6 cells/ml for 6 hours in the presence or absence of bacteria. It was previously determined that 6 hours represents an optimal time-point for cytokine mRNA synthesis by mouse adherent cells in response to LPS. Bacteria were added at different concentrations ranging from 10^9 to 10^7 cfu/ml. Bacteria were grown and stored as indicated above (page 6).

At the end of the 6-hour culture period, cells were isolated by centrifugation and lysed using the TRIzol reagent kit (GibcoBRL, Cat. No. 15596-018) following the manufacturer's instructions. Total RNA was isolated by isopropanol precipitation and reverse-transcribed into cDNA for 90 min at 42°C using 200 U reverse transcriptase (Superscript II, BRL) in a 40-μl reaction volume containing 200 mM Tris pH 8.3, 25 mM KCl, 1 μg/ml oligo $d(T)_{15}$ (Boehringer Mannheim), 1 mM DTT (Boehringer Mannheim), 4 mM of each dNTP (Boehringer Mannheim) and 40 U/ml Rnasin (Promega). PCR primers and conditions were used as already described in Kopf et al. (Journal of Experimental Medecine 1996 Sep 1;184(3):1127-36). Amounts of cDNA were normalized within the samples using primers specific for a house-keeping gene (β-2-microglobulin). PCR products were separated on a 2% agarose gel and bands were analyzed under UV.

As shown in Figure 4, ST11 showed the strongest induction of IL-12 and IL-10 mRNA, which was comparable to levels observed with the positive control (E. coli). Differences are best seen at the lowest bacteria concentrations (10^7 cfu/ml).

Example 6

Suppression of IL-4 synthesis by ST11

$CD4^+$ T cells were purified from the spleen of specific pathogen-free BALB/c mice using the MiniMACS kit from Miltenyi Biotec (Cat. No. 492-01). The $CD4^+$ T cells were cultured at a concentration of $2x10^5$ cells/ml in RPMI medium containing 10% fetal bovine serum, 1 mM L-Glutamine, 12 mM Hepes, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin and activated during one week by cross-linking with plate-bound monoclonal antibodies to CD3 (clone 2C11) and CD28 (clone 37.51, both antibodies from Pharmingen). During this 1ry culture, the $CD4^+$ T cells were co-cultured with bone marrow adherent cells (isolated as described above) as accessory cells and with 10^8 cfu/ml ST11, or 10^8 cfu/ml La1, or 1 mg/ml LPS, or medium alone. After this time, the cells were washed and $CD4^+$ T cells were

purified once again using MiniMACS kit technology and restimulated in a 2ry culture containing medium alone. Cytokines produced by the differentiated $CD4^+$ T cells were measured in the supernatants after 2 days using sandwich ELISA (kits from Endogen and Pharmingen).

Results are shown in Fig. 5. Cells differentiated in the presence of medium alone displayed a dominant Th2 phenotype characterized by high levels of IL-4. Addition of ST11 to the 1ry cultures strongly modulated the outcome of Th2 differentiation, as it resulted in an 8-fold decrease in IL-4 production. This inhibition was of similar magnitude as that observed in cultures derived from cells differentiated in the presence of LPS. In contrast thereto, the other Lactobacillus strain had no measurable impact on IL-4 levels. Interestingly, IFN-γ levels were not increased upon addition of ST11 in the 1ry cultures.

As may be seen from the above the strains of the present invention may be well prepared for the production of a food and/or a pharmaceutical carrier taking advantage of the valuable properties of the microorganisms.

<u>Example 7</u>

The ST11 strain was tested in a clinical trial in a community on the outskirts of Guatemala City on its ability to influence the transmission and experience of acute rainy-season diarrhoeal disease experienced by most of the children in this area. A total of 203 children, aged 35 to 70 months were enrolled in the study and received a target dose of 10^{10} viable organisms (ST11) or none (placebo) over a feeding period of 29 days. The children selected for both sample and placebo, respectively, had the typical deficits in weight-for-age and height-for-age characteristic due to undernutrition.

Before initiating the feeding trial in preschool children, a safety evaluation was conducted based on *in vitro* and *in vivo* studies. In vitro studies showed antibiotic resistance pattern similar to those of other lactobacilli used in food applications, and no potential for forming

biogenic amines, for degrading mucin and for deconjugating bile salts. In a placebo-controlled clinical study involving 42 adult volunteers, ST11 was well tolerated and did not induce any adverse effects, among the potential manifestations monitored, such as flatulence, number of stools per day and stool consistency; the levels of acute-phase proteins in serum did not raise any concern with respect to a potential inflammatory reaction.

The samples and placebo's have been packed in sachets at the Nestlé Product Technology Center manufacturing facility in Konolfingen, Switzerland, and shipped refrigerated to Guatemala. Each 10 g sachet consisted of a chocolate flavored vehicle and either 0.2 g of ST11 (10^{10} cfu) or, in case of the placebo, 0.2 g milk powder. The chocolate flavored vehicle consisted of cocoa powder, sugar, soy lecithin, vanillin, and cinnamon. The sachets were stored at 4° to 6°C until two hours before use. Before use the sachet had to be dissolved in 100 ml water provided by Nestlé, which was free of any bacterial contamination.

According to the protocol applied diarrhoea was defined as the occurrence of three or more liquid or unformed fecal evacuation during a period of 24 hrs. A diarrhoeal episode was defined as an event that presented the evidence of diarrhoea (3 diarrhoeal evacuations over 24 hrs). Its total duration in hrs was calculated from the moment of the first of the three index stools to the appearance of the first formed stool or a period 24 h without any defecation. For a child to have a "new" episode, 48 hours had to have elapsed the termination of the prior episode. If not, it was considered a continuation of the same episode and the total duration was then used for evaluation. A case was a child who experienced one or more documented episodes of diarrhoea through the 29-day observation period. Intensity of a diarrhoeal episode was based on the total number of loose stools produced. The elements of severity of the episode embraced the presence of blood, mucus or pus in stools, along with symptoms of fever and vomiting. An intensity of 7 stools per 24 h or the need for intervention by a health professional at a clinic, health center or hospital also classified an episode as severe.

When a diarrhoeal episode was diagnosed through the surveillance system, a specimen of a diarrhoeal stool was collected for microscopic examination and culture to identify potential

etiological pathogens for that episode. The specimen was diagnosed for rotavirus antigen, Giardia, and E. histolytica, in case the sample was dysenteric, and for bacterial pathogens including Shigella, Salmonella, Aeromonas, Plesiomonas shigelloides, E. Coli and possibly V. cholerae.

During the period of investigation product samples were collected to examine the viability of the microorganisms included during the period of administration. It could be shown that the microorganism stayed viable in the sachets during the entire study so that also at the end of the study the sachets were capable to convey 10^{10} viable microorganism upon reconstitution with water.

The study revealed that the sample containing the probiotic microorganism could decrease the occurrence of diarrhoea in contrast to the control group (placebo) by about 30 %. Yet, also the control group already exhibited a decreased number of diarrhoeal occurrence over the normal population receiving none of the samples or placebo, respectively. This latter finding may in part be explained on the basis of the children receiving additional valuable nutrition and contamination free water. However, since the study was performed in the field it may clearly be derived that ST11 can surely reduce the occurrence of diarrhoea in vivo.

TRAITE DE BUDAPEST ~~UR LA RECONNAISSANCE~~
INTERNATIONALE DU DEPOT DES MICRO-ORGANISMES
AUX FINS DE LA PROCEDURE EN MATIERE DE BREVETS

FORMULE INTERNATIONALE

DESTINATAIRE :

SOCIETE DES PRODUITS NESTLE S.A.
Patents department
Avenue Nestlé 55
CH-1800 Vevey

NOM ET ADRESSE
DU DEPOSANT

RECEPISSE EN CAS DE DEPOT INITIAL,
délivré en vertu de la règle 7.1 par
l'AUTORITE DE DEPOT INTERNATIONALE
identifiée au bas de cette page

I. IDENTIFICATION DU MICRO-ORGANISME

Référence d'identification donnée par le DEPOSANT :	Numéro d'ordre attribué par l'AUTORITE DE DEPOT INTERNATIONALE :
NCC 2461	**I - 2116**

II. DESCRIPTION SCIENTIFIQUE ET/OU DESIGNATION TAXONOMIQUE PROPOSEE

Le micro-organisme identifié sous chiffre I était accompagné :

■ d'une description scientifique

■ d'une désignation taxonomique proposée

(Cocher ce qui convient)

III. RECEPTION ET ACCEPTATION

La présente autorité de dépôt internationale accepte le micro-organisme identifié sous chiffre I, qu'elle a reçu le **12 JANVIER 1999** (date du dépôt initial) [1]

IV. RECEPTION D'UNE REQUETE EN CONVERSION

La présente autorité de dépôt internationale a reçu le micro-organisme identifié sous chiffre I le (date du dépôt initial)
et a recu une requête en conversion du dépôt initial en dépôt conforme au Traité de Budapest le (date de réception de la requête en conversion)

V. AUTORITE DE DEPOT INTERNATIONALE

Nom : **CNCM** Collection Nationale de Cultures de Microorganismes Adresse : **INSTITUT PASTEUR** 28, Rue du Docteur Roux F-75724 PARIS CEDEX 15	Signature(s) de la (des) personne(s) compétente(s) pour représenter l'autorité de dépôt internationale ou de l'(des) employé(s) autorisé(s) : **Mme Y. CERISIER** Directeur Administratif de la CNCM Cerisier Date : Paris, le 12 février 1999

1 En cas d'application de la règle 6.4.d), cette date est la date à laquelle le statut d'autorité de dépôt internationale a été acquis.

Claims

1. Lactic acid bacterium belonging to the genus Lactobacillus capable of adhering to and essentially colonizing the intestinal mucosa, capable of preventing infection of intestinal epithelial cells by rotaviruses and capable to grow in the presence of up to 0.4 % bile salts.

2. The Lactobacillus according to claim 1, which is selected from the group consisting of Lactobacillus rhamnosus or Lactobacillus paracasei.

3. The Lactobacillus according to claim 2, which is Lactobacillus paracasei.

4. The Lactobacillus paracasei strain according to claim 3, which is Lactobacillus paracasei CNCM I-2116.

5. Use of a lactic acid bacterium according to any of the preceding claims for the preparation of a ingestable support material.

6. The use according to claim 5, wherein the Lactobacillus strain is contained in the support material in an amount from about 10^5 cfu / g to about 10^{12} cfu / g support material.

7. The use according to any of the claims 5 or 6, wherein the support material is a food composition selected from milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formulae.

8. The use according to any of the claims 5 to 6, wherein the support material is used for the treatment and/or prophylaxis of disorders associated with diarrhea.

9. Food or pharmaceutical composition containing at least one lactic acid bacterium according to any of the claims 1 to 3.

10. The composition according to claim 9, which is selected from milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formulae, tablets, liquid bacterial suspensions, dried oral supplement, wet oral supplement, dry tube feeding or wet tubefeeding.

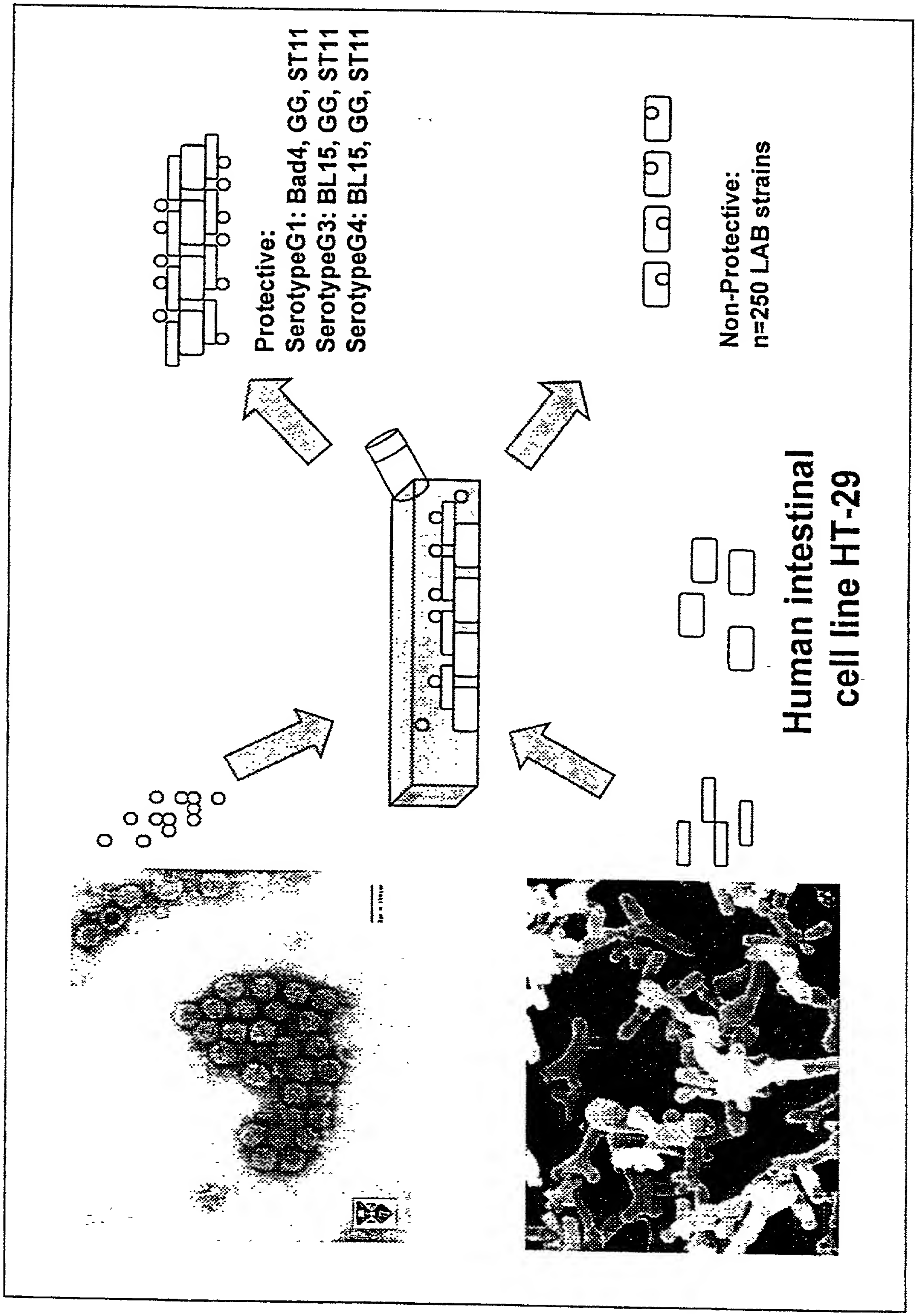

Protective:
SerotypeG1: Bad4, GG, ST11
SerotypeG3: BL15, GG, ST11
SerotypeG4: BL15, GG, ST11
Non-Protective:
n=250 LAB strains
Human intestinal
cell line HT-29

Fig. 1

Fig. 2

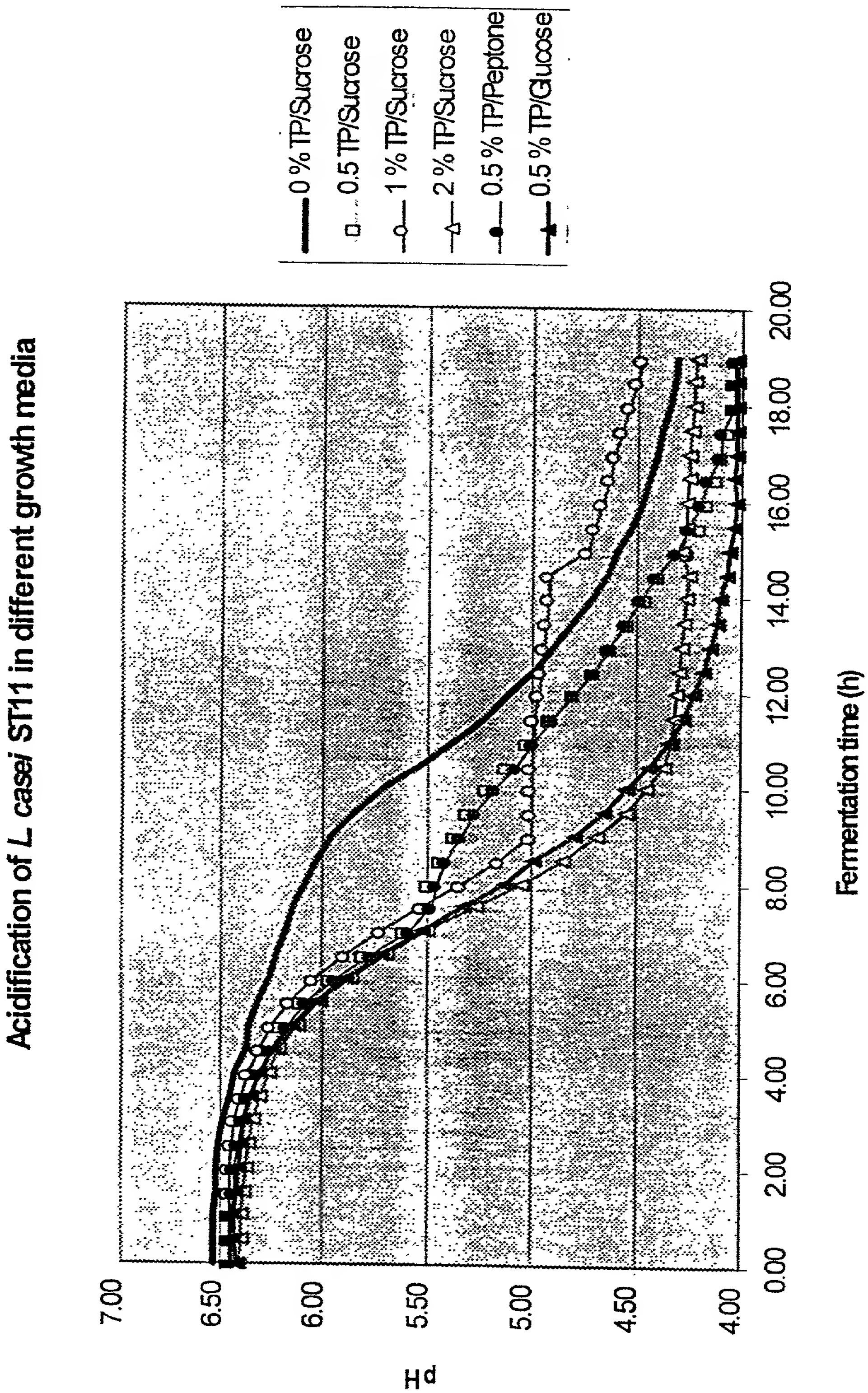
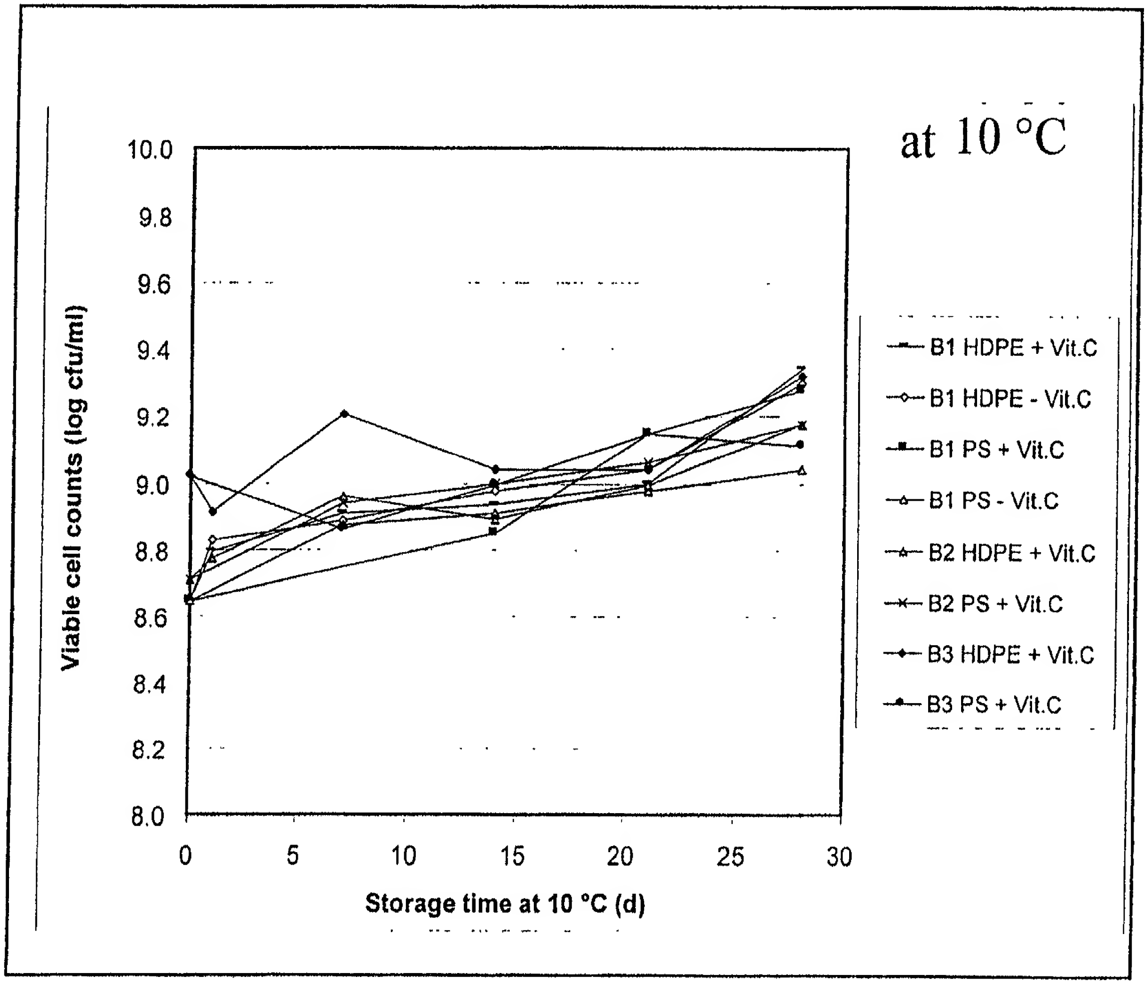

Acidification of *L casei* ST11 in different growth media
0 % TP/Sucrose
0.5 TP/Sucrose
1 % TP/Sucrose
2 % TP/Sucrose
0.5 % TP/Peptone
0.5 % TP/Glucose
pH
7.00
6.50
6.00
5.50
5.00
4.50
4.00
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Fermentation time (h)

Fig. 3

Survival in cereal drink



HDPE: High density poly ethilene PS: polystyrene Vit C: vitamin C

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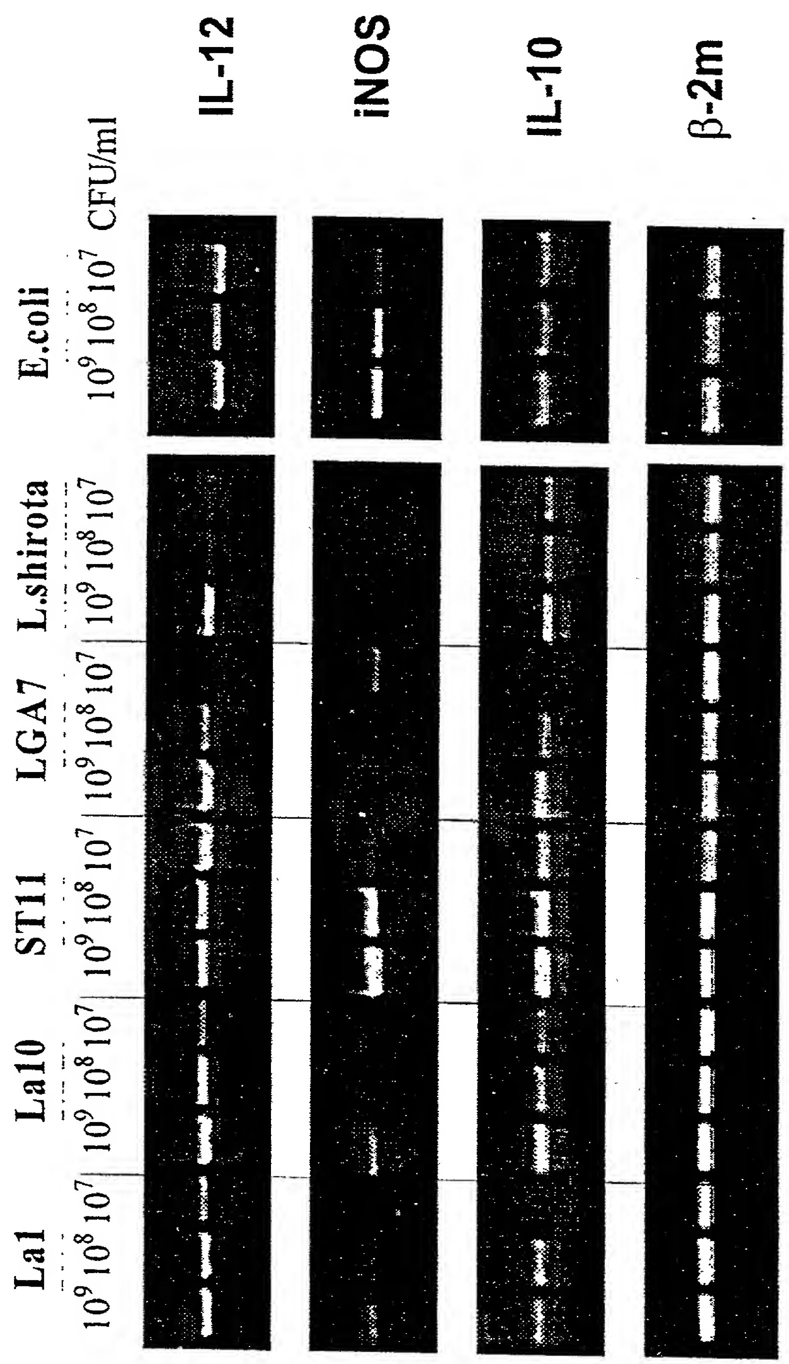
PCT/EP00/01797

Fig. 4 Induction of cytokine mRNA by LAB in mouse macrophages



09/936543

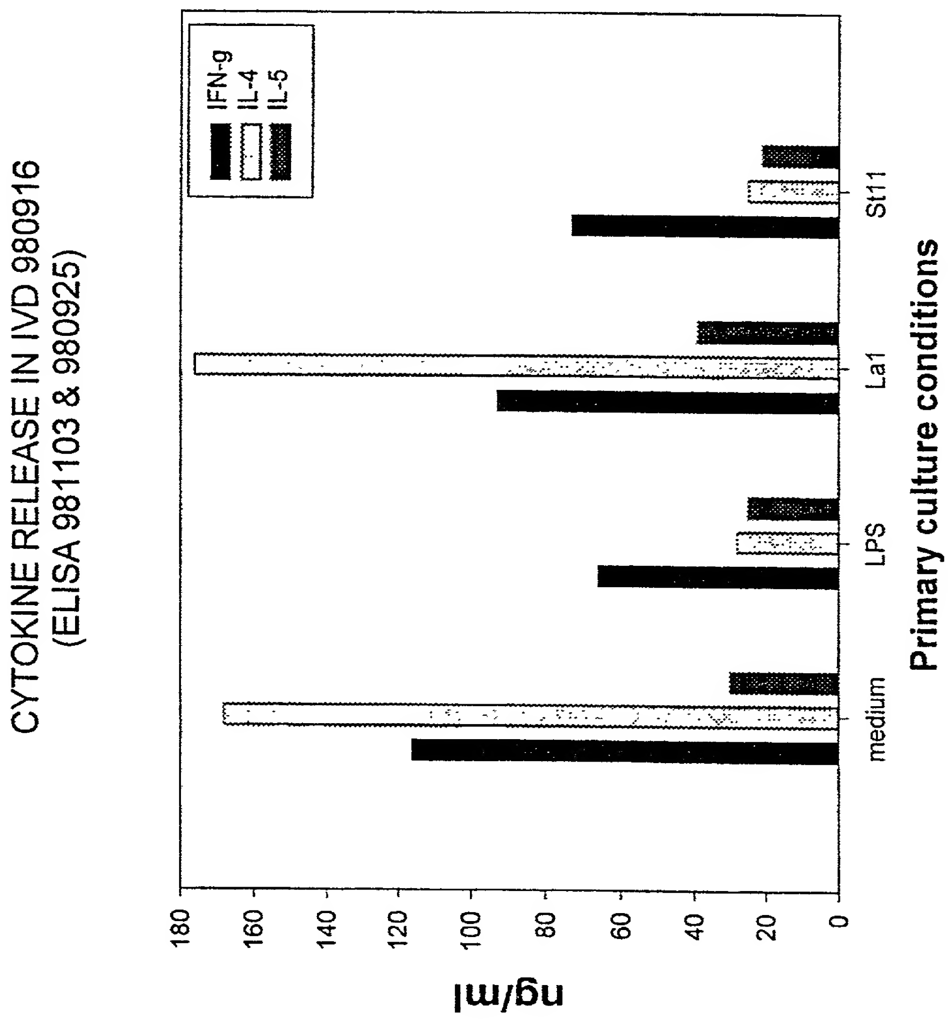


Fig. 5

Docket No.
112843-031

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled
LACTIC ACID BACTERIA STRAINS CAPABLE OF PREVENTING DIARRHOEA

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on 2 March 2000 as United States Application No. or PCT International Application Number PCT/EP00/01797
and was amended on February 28, 2001 and September 10, 2001
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

(Number)	(Country)	(Day/Month/Year Filed)	Priority Not Claimed
PCT/EP00/01797	WIPO	2 March 2000	☐
99104924.8	Europe	11 March 1999	☐
			☐

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

29174

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